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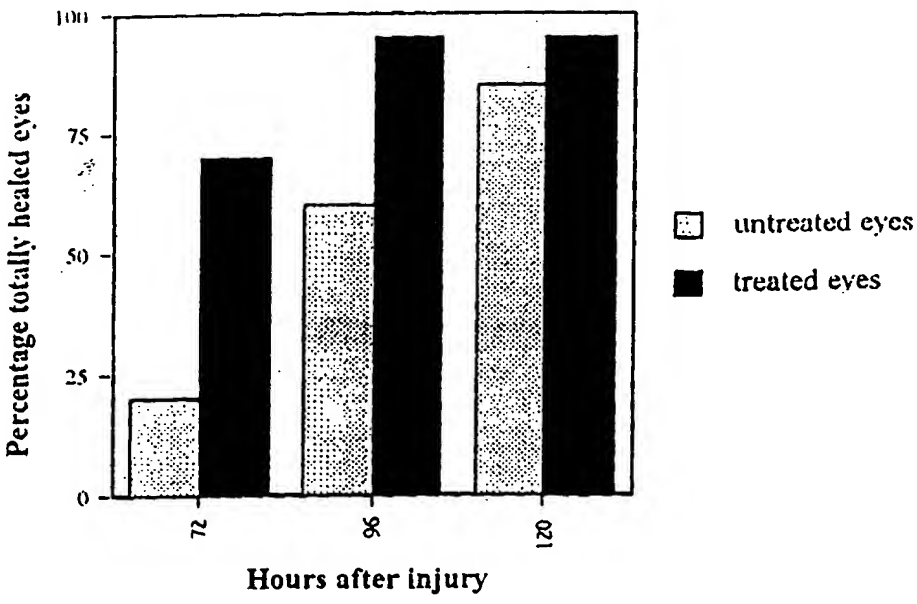

(57) Abstract

The invention relates to colagen webs which are formed *in situ* by dehydration and cross–linking of the insoluble collagen at sites of injuries, and to uses thereof in stimulating epithelial migration to assist wound healing.

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CROSS-LINKING OF COLLAGEN *IN-SITU* AND USES THEREOF IN WOUND HEALING

This invention relates to collagen-based materials, particularly an insoluble cross-linked collagen web, formed by dehydration of a colloidal suspension of insoluble collagen without use of a cross-linking agent or radiation. In particular, the invention relates to a web of collagen formed on tissue surface by *in situ* dehydration, and which is useful for stimulating wound healing, tissue repair or for delivery of pharmaceutically or therapeutically active agents. The collagen web according to the invention may also be formed *in situ* at nerve endings to provide an analgesic effect.

Background of the Invention

Collagen-based materials are used in a wide variety of ways in surgical procedures. These include providing a scaffolding for skin regeneration, dental films for the regeneration of periodontal growth tissues and the prevention of epithelial downgrowth on to the root of the tooth, collagen-based artificial ligaments such as anterior cruciate ligaments (ACLs), and injectable gels for tissue augmentation in plastic surgery. Collagen films are also used to prevent adhesions after surgery, and collagen composites are used to encourage bone growth.

Collagen is remodelled by tissue enzymes in the same way as other body tissues, and collagenases specific for various collagen types are found in many tissues.

The use of collagen in biomedical applications is reviewed in "Collagen as a biomaterial" in *Current perspectives in implantable devices*, Vol. 2, JAI Press Ltd, 1990, pp. 157-220, editors: J.A.W. Ramshaw, J.A. Werkmeister and D.E. Peters and "Biology, biotechnology and biocompatibility of collagen" in *Biocompatibility of tissue analogues*, CRC Press Boca Raton, 1985, editors: D.F. Williams and E.E. Sableman. Collagen preparations have

most commonly been used for soft tissue augmentation in cosmetic surgery.

Various forms of collagen preparations comprising either one or both of cross-linked or non-cross-linked collagen have been used to assist wound healing in various tissues, including the eye. For example, in US 4981841, a composition for enhancing the healing of deep wounds in the corneal stroma and for augmenting vision correction after keratorefractive surgery is described. The composition comprises an extracellular matrix such as fibronectin or collagen and an ophthalmologically compatible carrier material such as physiological saline. This composition may be further combined with other extracellular matrix materials such as chondroitin sulphate and a growth factor such as epidermal growth factor (EGF). This composition is inserted into the incision created during keratorefractive surgery.

In other preparations, the viscosity of the collagen preparations have been altered to enhance the injectability of the material into target sites for augmentation of soft tissue. For instance, US 5428024 describes vigorous mechanical disruption of both cross-linked and non-cross-linked collagen to reduce the average collagen fibre size below a threshold level. This enhances the injectability of a highly concentrated collagen preparation.

Another preparation used for augmenting connective tissue is disclosed in US 3949073. This preparation comprises an enzyme-solubilized collagen to which a polymerisation promoter has been added. On being warmed to physiological temperature at a target site, the preparation polymerises *in situ* into a stable, non-reactive fibrous mass of matter that is rapidly colonised by host cells and vascularised.

In US 4233360, there is disclosed a preparation of collagen which is soluble in dilute acidic aqueous solutions. The soluble collagen is first obtained under

conditions that are neutral to alkaline and which separate the protein slowly from the solution to form a precipitate. The precipitate is solubilized before undergoing treatment to form a fraction composed of regularly ordered fibres of collagen possessing rope-like structures known as native fibrous micropolymers (NFM). The optically-clear preparation was used to obtain a burn dressing comprising a film containing both cross-linked and non-cross-linked NFM which is laminated to a foam of NFM.

Suspensions of a mixture of collagen and a polysaccharide such as glycosaminoglycan have been demonstrated in US 4808570 to be effective in stimulating wound healing, compared with collagen preparations alone, particularly for ulcers in the joints and extremities of the limbs. The collagen in the mixture is present in a suspension at a concentration of 7 to 10 mg/ml.

The collagen preparations described above are preparations of soluble collagen or insoluble collagen which may be cross-linked before use. Cross-linking is achieved by a variety of methods, but is always performed in the aqueous phase. Some of these preparations are further cross-linked *in situ*. This requires the use of additional cross-linking agents such as formaldehyde or glutaraldehyde, which are undesirable because of their toxicity.

Cross-linking may also be achieved by radiation. For example, collagen lenticules described in US 5288436 are bonded to a patient's cornea, to provide the refractive changes necessary for vision correction. The collagen is cured or cross-linked *in situ* by irradiation or exposure to UV, infra-red or gamma rays. Cross-linking of collagen in this manner is disadvantageous and dangerous; certain wavelengths are particularly undesirable because of the potential mutagenic effects on living cells. Furthermore, the integrity of the collagen may be affected. Thus, it would be beneficial to obtain a collagen base for wound healing or tissue re-generation which does not require use

of toxic agents or radiation.

Normal epithelial healing of the eye depends on healthy corneal components such as the basement membrane and the overall ocular environment, as well as sufficient lubrication. Where such conditions are absent, medical intervention is necessary, and current methods of treatment of persistent epithelial defects include patching, bandage lenses, ointments, microstromal puncture, and laser treatment. All these forms of treatment have drawbacks which include the need for long term use and possible iatrogenic effects.

Thus, it would be extremely advantageous to have a simple, safe treatment that can be administered in the consulting room, for example in the form of eyedrops which stimulate epithelial regrowth, attachment of the epithelium and which can also reduce pain in the short term.

There is also a need to increase the "persistence" of collagen implants, persistence being defined as the ability of the implant to form a cohesive mass and resist migration from the target site.

While the invention is described particularly in relation to the treatment of ocular conditions, it will be clearly understood that the invention encompasses other embodiments not limited to use at this site.

Summary of the Invention

In one aspect, the invention provides a cross-linked collagen web formed on a tissue surface by *in situ* dehydration of a colloidal suspension of insoluble collagen on said tissue surface.

The colloidal suspension of the invention is cross-linked by drying, and remains insoluble when hydrated. Drying is the only requirement for cross-linking on the surface to which the suspension is applied. No other agents are necessary. After drying, an insoluble collagen mat is formed. This mat, when rehydrated by exposure to physiological fluids in the tissue site, is

relatively stiff, and forms a surface suitable for epithelial migration. The suspension is non-toxic, and avoids the need to use toxic cross-linking agents such as formaldehyde or glutaraldehyde. Undesirable irradiation is also avoided.

The suspension of insoluble collagen according to the invention may be applied to areas of injured skin or other tissues, but preferably to an ocular surface or nerve endings in the eye. For example, the preparation may be applied to an injured cornea using a template such as a contact lens, and dried quickly with a sterile stream of air. This may be required in cases of deep injury or pathology.

The web of cross-linked collagen can also be formed on any corneal abrasion, site of injury or damage where the epithelium is absent or disrupted. In one embodiment, an insoluble rigid web or mat of cross-linked collagen is formed across an exposed stromal surface to provide a support for epithelial migration, thus assisting healing or closure of the wounded stroma. Thus, the suspension of insoluble collagen may be used in the treatment of eye injuries, particularly when epithelial healing is poor or is expected to be poor. Examples of such injuries include, but are not limited to, burns in the eye caused by acid, alkali or other chemicals, viral or bacterial infection, basement membrane injury or other forms of ocular trauma, as well as any injury which may cause morbidity and pose a risk to the sight.

The insoluble cross-linked collagen web according to the invention provides a rigid base which supports migration of the epithelium. During the formation of cross-links in the collagen during the drying process, cross-linking to the exposed stromal surface may also occur, for example to the exposed, de-epithelialized surface of the cornea. This assists in the anchoring of the mat or web in accordance with the invention to the site of injury, and stimulates wound healing.

The ability of the collagen web to support cell migration also renders it particularly useful in repair of injured body surfaces where epithelial closure can be accelerated by provision of this collagen composition as a replacement basement membrane structure. Epithelia may be stratified, simple or pseudostratified. Mucosal surfaces such as those of the cervix and bronchial passages may be particularly suited to this procedure. Thus, the invention is useful in treatment of dermal injuries such as dermal ulceration, including varicose and diabetic ulcers, and in repair of mucosal surfaces such as those of the bronchi, or the buccal mucosa. For example, the collagen web is suitable for use after removal of tumours or after colposcopic surgery. The web may also be used to treat aphthous ulcers, herpetic ulcers or the like.

Therefore, in a second aspect, the invention provides a method of assisting wound healing, comprising the step of forming an insoluble cross-linked collagen web to support cell or epithelial migration, said web being formed on a wound site by *in situ* dehydration of a colloidal suspension of insoluble collagen on said site.

In a third aspect, the invention provides an analgesic composition comprising a colloidal suspension of insoluble collagen, which suspension forms an insoluble cross-linked collagen web on exposed nerve endings to cover the nerve endings thereby to reduce pain stimuli. Optionally, the composition additionally comprises another analgesic or local anaesthetic agent.

The insoluble collagen is preferably substantially derived from a nature Type I collagen, and may be an atelopeptide collagen. The collagen may be derived from animal or human tissue, or can be synthesised by recombinant methods.

The colloidal suspension of insoluble collagen which is to be applied to a target tissue surface is preferably prepared by mechanical dissociation or shearing of the collagen fibrils to form a colloid. A uniform,

viscous colloid may then be isolated by removing particulate matter following centrifugation. The viscosity of the colloidal suspension can be adjusted with solvent such that it can be accurately applied to an abrasion or site of injury by a fine-gauge tip or fine pipette, and can spread to the margins of the treated area quickly. The colloidal suspension dries down quickly on the tissue surface to form a very thin web, preferably less than 10μ, most preferably 0.5-1μ.

A 10 μl aliquot is normally sufficient to provide coverage over an epithelial shallow wound of approximately 50mm^2 in area. Air drying is sufficient for surface wounds, but other defects may require drying in a sterile air stream. The drying time is generally 1 to 2 minutes.

The dose to be administered will vary with the condition to be treated, the extent of the injury and will be easily determined by a person skilled in the art.

The colloidal suspension may also be applied to the surface under sterile conditions using a routine of dehydration and rehydration to ensure firm attachment of the mat to the target surface. This is specifically useful for creating a surface coating suitable for epithelial migration on top of a rough keratoprosthesis surface.

Application of the suspension in this manner may also be useful in tissue culture applications.

The efficacy of the product may be determined by examination of epithelial closure, patient report on the reduction of pain, by long-term response and quality of healing.

When the insoluble collagen web forms on the site of the injury or wound, the collagen web can also act as a delivery vehicle for pharmaceutical or therapeutic agents. Therefore, the colloidal suspension of insoluble collagen may comprise one or more therapeutic agents specific to the healing of wounds, or analgesic or local anaesthetic agents. Preferably these agents are especially suitable for topical application, but agents suitable for systemic

use may also be used. These include, but are not limited to, compounds with anti-inflammatory properties such as steroids, local anaesthetics such as novocaine or neurocaine, analgesics such as paracetamol, antibiotics such as neomycin sulphate, or antiviral agents such as a cyclovir or ganciclovir. Promoters of wound healing such as fibroblast growth factor (FGF), transforming growth factor β (TGF) and epithelial growth factor (EGF) may also be included. Similarly, other extracellular matrix or basement membrane components may also be added to the starting material. Preferably these include, but are not limited to proteoglycans, glycosaminoglycans, fibronectin, laminin, and collagens IV and VII. Film-forming agents such as hyaluronic acid and polyethyleneglycol may also be included. Collagenase inhibitors such as tissue inhibitors of metalloproteinases which would prevent breakdown of collagen when the wound is healing may also be incorporated. However, chelating agents which will inhibit integrins or formation of extracellular matrix should preferably not be used.

Thus, in a fourth aspect, the invention provides a method of delivering a pharmaceutically active agent to a target site, comprising administration of a colloidal suspension of insoluble collagen comprising one or more of said agents to the target site thereby to allow dehydration of said suspension to form a collagen web and to deliver said agent. The target site is preferably a tissue surface which may be a dermal surface but is preferably an ocular surface and most preferably, a stromal surface.

In addition to increasing the speed of wound closure by encouraging epithelial cell migration, the suspension of insoluble collagen according to the invention may also be used to provide a coating on synthetic materials for use in the eye, or for other prosthetic use. In this way, the synthetic surface coated with the preparation of invention can be used to re-construct various structural surfaces. However, it is to be

understood that the present invention is not designed as a permanent epikeratoprosthesis or to provide refractive correction.

Some injuries and pathologies of the ocular surface require that epithelial tissue be grafted to the damaged area from an undamaged part of the same, or contra-lateral eye. In severe conditions, very little undamaged epithelium is available, thus making transplantation difficult. The expansion of very small amounts of epithelium in culture has been observed. However, a suitable culture support has not been provided.

The collagen suspension or formulations of the invention can also be used as a cell culture support *in vitro* for autologous corneal epithelial tissues. Thick films (20-100 microns thick) may be cast on to polystyrene culture surfaces and dried under sterile conditions. The formulations may include other agents eg. therapeutic agents as described above. In a preferred embodiment, corneal cells are harvested from a small section of undamaged human cornea, and expanded *in vitro* on a culture support comprising of the insoluble collagen film. The culture is allowed to grow to the required surface area, and then transferred back to the patient's damaged corneal surface. The collagen support integrates well with the patient's ocular surface, and provides a means of suturing the epithelium transplant in place.

Accordingly, in a fifth aspect, the invention provides an explant comprising a cross-linked collagen web formed on a support by *in-situ* dehydration of a colloidal suspension of insoluble collagen on said support. In a particularly preferred embodiment, the explant further comprises viable cells such as epithelial cells from the cornea, skin or any other derived tissues. The explant can then be transferred back to the host or to a patient as an epithelium transplant.

Detailed Description of the Invention

The description will now be described by way of example only, with reference to the following figures in which:-

Figure 1 shows the rate of healing of epithelial defects in rabbit cornea treated with a suspension of insoluble collagen I in acetic acid, compared with untreated cornea.

Figure 2 represents the results of a stress viscometry test on a colloidal suspension of insoluble collagen using a C5-10 Bohlin Rheometer.

Figure 3 shows the results of an oscillation test on the colloidal suspension of Figure 2, using a C5-10 Bohlin Rheometer.

Figure 4 is a light micrograph of frozen section of a collagen mat (dark area) formed on top of a polymer sponge (lighter areas). The depth of the coating is between 1-10 microns and interpolation into the sponge layer can be seen.

Figure 5 is a light micrograph of a section (stained with toluidine blue) showing the epithelial outgrowth onto a polymer sponge coated with the collagen formulation. The epithelial sheet has migrated down the side of the explant (on the left) and across the surface of the coating on the polymer sponge. Epithelial migration does not occur in the absence of the coating on this particular polymer.

Figure 6 is a plan view fluorescent image of an epithelial cell sheet outgrowth onto a collagen mat from a corneal explant.

Figure 7 shows the beneficial effects of insoluble collagen formed *in situ* on healing of corneal epithelial defects caused by alkali burns in rabbits, compared with treatment with chloramphenicol alone.

Figure 8 shows the rate of healing of burn injuries of the corneal epithelium in rabbits treated with chloramphenicol or chloramphenicol + insoluble collagen.

Example 1: Preparation of Colloidal Suspension of Insoluble Collagen

60 mg of insoluble Type I Collagen obtained from bovine skin was mechanically dissociated by homogenisation in 20 ml of 50 mM acetic acid under sterile conditions to form a colloid. The resulting homogenate was centrifuged at 24000 rpm for 5 minutes. The acetic acid protonates the collage fibrils, resulting in formation of a stable colloid. This in turn allows for uniformity of coating and for good cross-linking during dehydration.

The bottom fraction, which consisted of about 30% of the total volume and contained particulate matter, was discarded. The viscosity of the supernatant comprising uniform, viscous colloid was adjusted by altering the initial concentration of collagen using solvent. The viscous colloidal suspension of acid insoluble collagen appeared opalescent. After drying onto a transparent surface (such as a tissue culture petri dish), the insoluble mat appeared slightly hazy. Only mechanical disruption would affect the nature of the structure.

Sterility of the colloidal suspension was ensured by the use of filter-sterilised acetic acid, and collagen which had been subjected to ultraviolet radiation. The suspension was prepared under sterile conditions, and the suspension remained in a sealed vessel until use by a clinician. Sterility tests were carried out in a standard microbiological testing environment.

The isolated product has a limited shelf life, and all suspensions must be stored at 4°C to avoid gelation. Conventional cryoprotectants such as glycerol and/or DMSO, novel cryoprotectant polymers, carbohydrates and sugars and/or preservatives may be included to prolong the shelf life of the preparation. For ocular applications it is preferable to avoid the use of glycerol, which tends to have a dehydrating effect on the cornea. Glucose, sucrose or other simple or complex sugars are preferable because of their suitability for application to the eye.

Soluble or lightly cross-linked polymers suitable for protection of proteins in solution, for example, polyvinylpyrrolidone (PVP), polyvinylacetate (PVA), polyethylene glycol or the like may be used.

Example 2: Rheological Properties of Colloidal Suspension of Insoluble Collagen

Oscillation tests using a Bohlin rheometer show that the colloidal suspension of insoluble collagen in acetic acid is a viscoelastic material, predominantly viscous, with an elastic component of approximately 30% (Figures 2 and 3). Stress viscometry shows that, after shearing, the viscosity of the material at 37C is approximately 4.2mPa. The viscosity of the material for clinical use may lie between 3.2 and 5.2 mPa.

Example 3: Effect of colloidal suspension of insoluble collagen on wound healing in rabbit cornea.

Circular corneal defects, 7.5 mm in diameter, were created by scraping areas in each of twenty rabbit corneas. Ten corneas were treated using 10 µl aliquots of the collagen preparation prepared in Example 1. The preparation was applied to the wounds with a fine tip pipette and allowed to dry. The remaining ten rabbits were untreated (controls). Healing was then allowed to proceed without further intervention, except that all animals received topical chloramphenicol once daily. The diameter of the epithelial defects was monitored each day, using fluorescein under an operating microscope.

Figure 1 demonstrates an increased rate of epithelial closure over the first two days on rabbit corneas that had been treated with the collagen preparation compared to the controls. No toxic effects were observed, and the healed corneas that had been treated with collagen did not appear different to those that were untreated.

Complications should be few after the use of this product. Mild corneal haze was expected to occur as the

product is not transparent, but was not found to be significant in animal trials. However, the haze disappeared over time as the collagen remodelled, and in any case caused no problems in non-ocular applications. Macrophage activation may also be another side effect of the application.

Example 4: ***In vitro* Epithelial Growth on Collagen Web**

Approximately 100 microlitre aliquots of the collagen suspension were applied to hydrogel sponges 1cm in diameter. The sponges were composed of poly (2-hydroxyethyl)methacrylate (PHEMA) with 5% methyl methacrylate (MMA) as a comonomer. After drying under sterile conditions, the sponge-collagen moieties were rehydrated in phosphate buffered saline. Frozen sections revealed that the collagen layer was interpolated into the rough surface of the sponge, as shown in Figure 4. The thickness of the mat varied between 1 and 10 microns.

Corneal buttons consisting of stroma and surface epithelia were placed on top of the collagen coated sponges, and incubated at 37C, 5% CO2 for 4 days in serum free medium (Hams F12 /DMEM 50:50). The epithelial sheet migrated down the side of the corneal button, and along the surface of the collagen sheet, as shown by the toluidine blue stained section illustrated in Figure 5. The explant and migrating sheet on the surface of the coated sponge was also viewed from the top surface using a live stain to visualise the cells. This plan view is shown in Figure 6.

In control experiments using uncoated sponges, no epithelial migration onto the sponge was noted.

Example 5: Effect of Colloidal Suspension of Insoluble Collagen on Wound Healing of Corneal Epithelia Following Alkali Burning.

The effects of *in-situ* formation of a web of insoluble type I collagen on wound healing in the cornea following injuries caused by alkali burning were studied.

40 rabbits (A1-A40) were divided into the following groups following alkali injury:-

1. treatment with chloramphenicol ointment,
2. treatment with chloramphenicol ointment and a colloidal suspension of insoluble collagen I.

Sterile insoluble collagen I was prepared as described in Example 1. Surgical procedures were performed in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes.

Alkali injury:

Under general anaesthesia and sterile preparation, the right eye of each animal was proptosed. An 8 mm trephine was used to punch out a sterile disc (0.5 mm thick) of a porous sponge (poly(2-hydroxyethyl methacrylate)) which was soaked in 1M sodium hydroxide solution for 5 minutes. This was then placed on the cornea, half way between the superior limbus and the centre, for 30 seconds. The sponge was then removed, and the cornea was irrigated with 10 ml of sterile saline for 30 seconds before the loose epithelium was wiped off. In the treated group (rabbits A21-A40), 10µl of sterile bovine collagen I solution was applied using a Biotip electronic micropipette, and allowed to dry at room temperature for 2 minutes. The lesion was stained with sodium fluorescein and photographed with a scale marker in place, and the eye was then allowed to return to the socket. All animals were then given chloramphenicol ointment and one drop of guttae atropine. At intervals of 24, 48, 72, 96 and 120 hours, the animals were returned to theatre under general anaesthesia and the epithelial lesions were again stained and photographed as transparencies. Each animal received chloramphenicol ointment once daily during the healing period, and no other treatment was given. At the last examination, at 120 hours, 5 treated and 5 untreated animals were euthanased

for histological examination. One month after the experiment, a further 5 treated and 5 untreated animals were euthanased for histology. One animal, A14, died under anaesthesia at 24 hours.

The transparencies were projected on to a vertical grid of 10 mm squares, such that the 1 mm divisions on the scale marker included in each photograph coincided with the 10 mm grid. Two independent observers, blind to the identities of the treated and untreated animals, counted the area of each lesion in square mm. Two separate photographs had been taken for each animal at each time point, so that a total of 4 estimates of surface area was available, and the mean of these was taken to be the area of the remaining epithelial defect.

The results are represented in Figure 7, and show that the percentage of totally healed corneas at 72 and 96 hours was significantly different between control (chloramphenicol only) and treatment (chloramphenicol + collagen film) groups (n=20, P=0.0003, P=0.0047 , t-test).

As shown in Figure 8, the rate of healing was increased significantly by collagen treatment. The upper and lower lines represent the rate of healing (in mm per hour) from 24-48 hours, in collagen treated, and control eyes respectively (P=0.0085, students t-test).

This study shows that the addition of an insoluble type I collagen film to corneal surfaces injured by alkali enhances the rate of epithelial healing, and significantly decreases the total time required to heal when compared with untreated eyes. The results from this study demonstrate the potential for clinical application of the collagen web of the invention.

The data from the studies described above show that the colloidal suspensions of acid insoluble collagen can be applied to surfaces and dried, whereupon an insoluble mat of highly cross-linked, insoluble collagen is formed without using cross-linking agents or irradiation.

Rehydration of this mat in the aqueous environment in the ocular region may cause swelling, but does not disturb the structure of the mat. The mat presents a rigid surface which supports corneal epithelial outgrowth or migration. It also provides a surface that resists the traction forces of a migrating epithelial sheet, unlike a gelled preparation. These features provide an advantage over the use of gelled preparations. The insoluble mat is re-modelled slowly, that is, it has persistence, or the tendency for the suspension to form a cohesive mass and resist migration from a target site.

Considerable traction is exerted by a migrating sheet on the surface across which it moves. Tension applied to the underlying surface actually stimulates migration (2). Epithelial migration is also regulated by a number of factors, including hyaluronic acid (3), proteases (4), plasmin (5), glycolytic enzymes (6), growth factors (7), matrix proteins such as fibronectin (Fn) (8,9) and innervation (10). Activation of matrix metalloproteases (MMPs) in the stroma and epithelium are an essential part of the wound healing process where tissue remodelling must occur. Once closure has occurred, the epithelium restratifies and produces basement membrane and HDs.

Wound closure precipitates a set of biological events that downregulate the production of MMPs in both the epithelium and stroma. Additionally, the stromal collagenases are no longer activated by tear proteases, so that collagenolytic processes are slowed.

In defective healing, the delayed closure of the epithelium ensures that proteolytic activation of both the leading edge of the epithelium and activated keratocytes in the stroma persists. Additionally, tear proteases activate stromal collagenases, and the complex system of collagenolytic degradation is maintained in an activated state (11). Degraded collagens no longer provide a stable surface for epithelial migration. Thus, continual breakdown, or persistent failure to heal may occur.

The need for a mechanically stable surface may be one of the most important factors in many of these pathological states. Critically, the epithelial sheet exerts considerable traction on the underlying surface, i.e. the basement membrane, the superficial stroma in the case of scraped epithelial, or a fibrin/fibronectin matrix in the case of exposure to laser radiation or burned ocular surfaces. The traction exerted is a result of the cytoskeletal stresses placed by each cell onto the substrate. Such mechanisms are easily observed in fibroblasts and epithelial cells that are contracting within collagen gels *in vitro* (12). Where considerable corneal stromal melting occurs, the mechanical stability of the underlying stroma may be insufficient for the complete migration and closure of the wound. The aetiology of persistent corneal epithelial lesions includes dystrophies, alkali injury and viral infection. Current management options include lubricants, antibiotic ointments, patching, bandage contact lenses, stromal micropuncture and epithelial debridement by blade or laser. The aim of treatment is to encourage epithelial coverage and attachment, so breaking the cycle of epithelial loss, enzyme release, inflammation and melting (13). Matrix proteins such as fibronectin (Fn), a matrix protein important in the epithelial healing process, have been applied exogenously, and variously found to increase or to have little effect on the healing rate. Current data suggests that applied Fn may be useful in cases where endogenous Fn is at low levels or chemically altered, as in the case of alkali burns (8,9). However, such matrix proteins are quickly digested by the activated collagenases present in many of the pathological states which cause persistent epithelial defects. Additionally, matrix proteins such as fibronectin and collagen IV do not provide mechanically stable coverage.

Collagen is used clinically in the form of degradable (soluble) collagen I bandage lenses. These

lenses protect exposed corneas, and can provide useful drug delivery and improved comfort post-operatively. Generally, such lenses are supposed to have little effect on the rate of healing, but do not inhibit it, and provide protection against pathogens at the ocular surface (14, 15). Insoluble collagen matrices are used as bases for the culture of keratinocytes for burn coverage (16) and as thicker, non-cellular sheets for the coverage of chronic leg ulcers and pressure sores, and in reconstructive surgery, where they appeared to improve wound repair (17).

Without wishing to be bound by any proposed mechanism, it is believed that the use of insoluble collagen on an injured area such as a corneal surface firstly provides a mechanically rigid surface for epithelial migration, that cross-links to the underlying stroma. Secondly, insoluble collagen does not degrade quickly under attack by the matrix metalloproteases that are upregulated in these lesions, in contrast to collagen gels, and other matrix proteins.

The invention also avoids the use of undesirable toxic cross-linking agents or the use of radiation.

Although the invention has been described with reference to the eye to provide a clear understanding of the invention, it is to be understood that it may be embodied in various other forms. Such embodiments would be within the knowledge of a person skilled in the art.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in the specification.

References cited herein are listed on the following pages, and are incorporated herein by this reference.

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CLAIMS

1. A cross-linked collagen web formed on a tissue surface by *in situ* dehydration of a colloidal suspension of insoluble collagen on said tissue surface.

2. A cross-linked collagen web according to claim 1, wherein the dehydration on the tissue surface is effected by air drying or by drying with a stream of air.

3. A cross-linked collagen web formed across an exposed stromal surface to provide a support for epithelial migration, wherein said web is formed by *in-situ* dehydration of a colloidal suspension of insoluble collagen on said surface.

4. A cross-linked collagen web according to any one of claims 1 to 3 formed on a corneal abrasion, site of injury or damage, where epithelium is absent or disrupted, to support migration of the epithelium.

5. A cross-linked collagen web according to any one of claims 1 to 4, further comprising a pharmaceutically or therapeutically active agent.

6. A cross-linked collagen web according to any one of claims 1 to 5, having a thickness of 10μm or less.

7. A collagen web according to any one of claims 1 to 6, wherein said insoluble collagen is a native type I collagen or an atelopeptide collagen.

8. A collagen web according to claim 7, wherein the collagen is from a source selected from the group consisting of animal tissue, human tissue and synthetic collagen prepared using recombinant methods.

9. A method of assisting wound healing, comprising the step of forming an insoluble cross-linked collagen web to support cell or epithelial migration, said web being formed on a wound site by *in situ* dehydration of a colloidal suspension of insoluble collagen on said site.

10. A method according to claim 9, wherein the wound site is in the eye.

11. A method according to claim 10, wherein the wound site is the cornea.

12. A method according to claim 11, wherein the web of cross-linked collagen is formed on a site of corneal abrasion and where the epithelium is absent or disrupted.

13. A method according to any one of claims 9 to 12, wherein the web of cross-linked collagen is an insoluble rigid mat formed across an exposed stromal surface to provide support for epithelial migration.

14. A method according to any one of claims 9 to 13, wherein the wound is caused by means selected from the group consisting of burns caused by acid, alkali, or other chemicals; viral infection; bacterial infection; basement membrane injury; and ocular trauma.

15. A method according to any one of claims 9 to 14, wherein the formation of the web further comprises cross-linking of the collagen to the wound site during the dehydration of the colloidal suspension of insoluble collagen.

16. A method according to any one of claims 9 to 15, wherein said insoluble collagen is a type I collagen or an atelopeptide collagen.

17. A method according to any one of claims 9 to 16, wherein the collagen is from a source selected from the group consisting of animal tissue, human tissue and synthetic collagen prepared using recombinant methods.

18. An analgesic composition comprising a colloidal suspension of insoluble collagen, which suspension forms an insoluble cross-linked collagen web on exposed nerve endings to cover the nerve endings thereby to reduce pain stimuli.

19. An analgesic composition according to claim 18, further comprising another analgesic or local anaesthetic agent.

20. A composition according to any one of claims 18 to 19, wherein said insoluble collagen is a type I collagen or an atelopeptide collagen.

21. A composition according to any one of claims 18 to 20, wherein the collagen is from a source selected from the group consisting of animal tissue, human tissue and synthetic collagen prepared using recombinant methods.

22. A method of delivering a pharmaceutically active agent to a target site, comprising administration of a colloidal suspension of insoluble collagen comprising one or more of said agent to the target site thereby to allow dehydration of said suspension to form a collagen web and to deliver said agent.

23. A method according to claim 22, wherein said active agent is selected from the group consisting of steroids, local anaesthetics, analgesics, antibiotics, anti-viral agents, growth factors, extracellular matrix components, film-forming agents and collagenase inhibitors.

24. A method according to claim 22 or claim 23, wherein said target site is a tissue surface selected from the group consisting of a dermal surface, an ocular surface and a stromal surface.

25. A method according to any one of claims 22 to 24, wherein said insoluble collagen is a type I collagen or an atelopeptide collagen.

26. A method according to any one of claims 22 to 25, wherein the collagen is from a source selected from the group consisting of animal tissue, human tissue and synthetic collagen prepared using recombinant methods.

27. An explant comprising a cross-linked collagen web formed on a support by *in-situ* dehydration of a colloidal suspension of insoluble collagen on said support.

28. An explant according to claim 27, further comprising viable epithelial cells.

29. An explant according to any one of claims 27 to 28, wherein said insoluble collagen is a type I collagen or an atelopeptide collagen.

30. An explant according to any one of claims 27 to 29, wherein the collagen is from a source selected from the group consisting of animal tissue, human tissue and synthetic collagen prepared using recombinant methods.

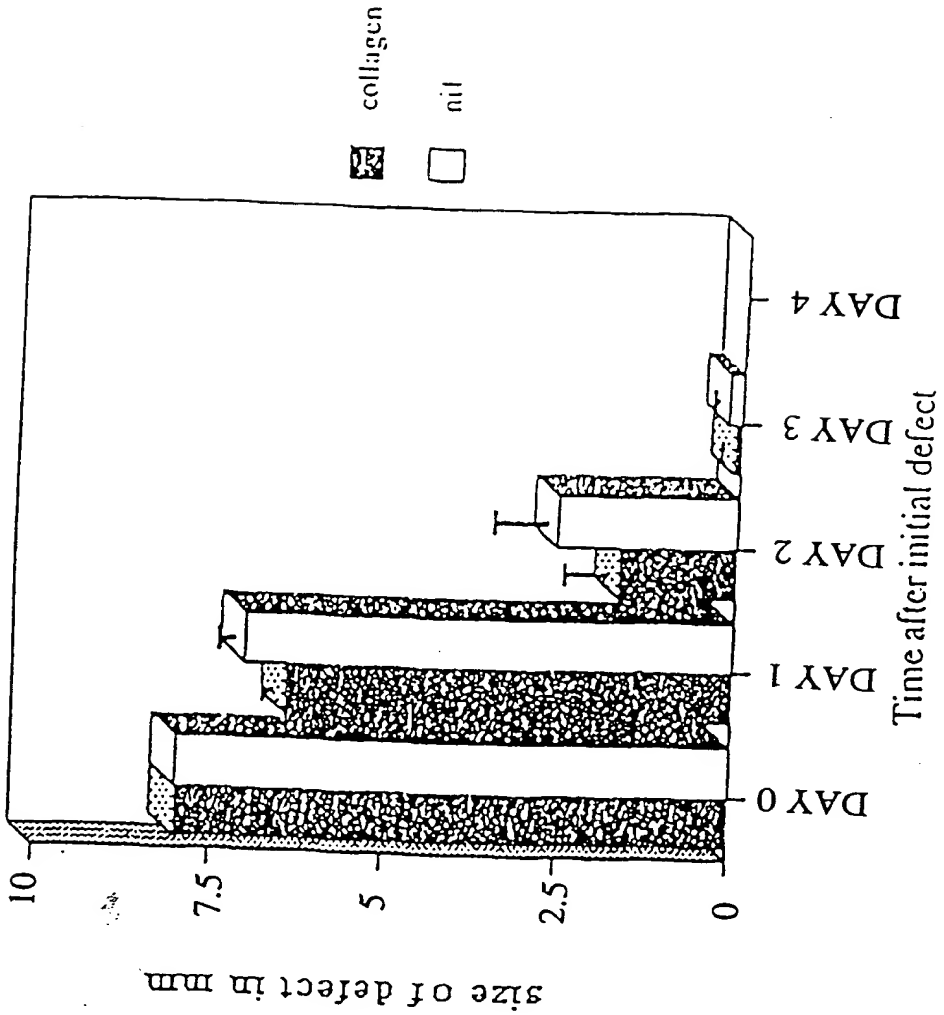

collagen
nil
DAY 4
DAY 3
DAY 2
DAY 1
DAY 0
Time after initial defect
10
7.5
5
2.5
0
size of defect in mm

FIGURE 1

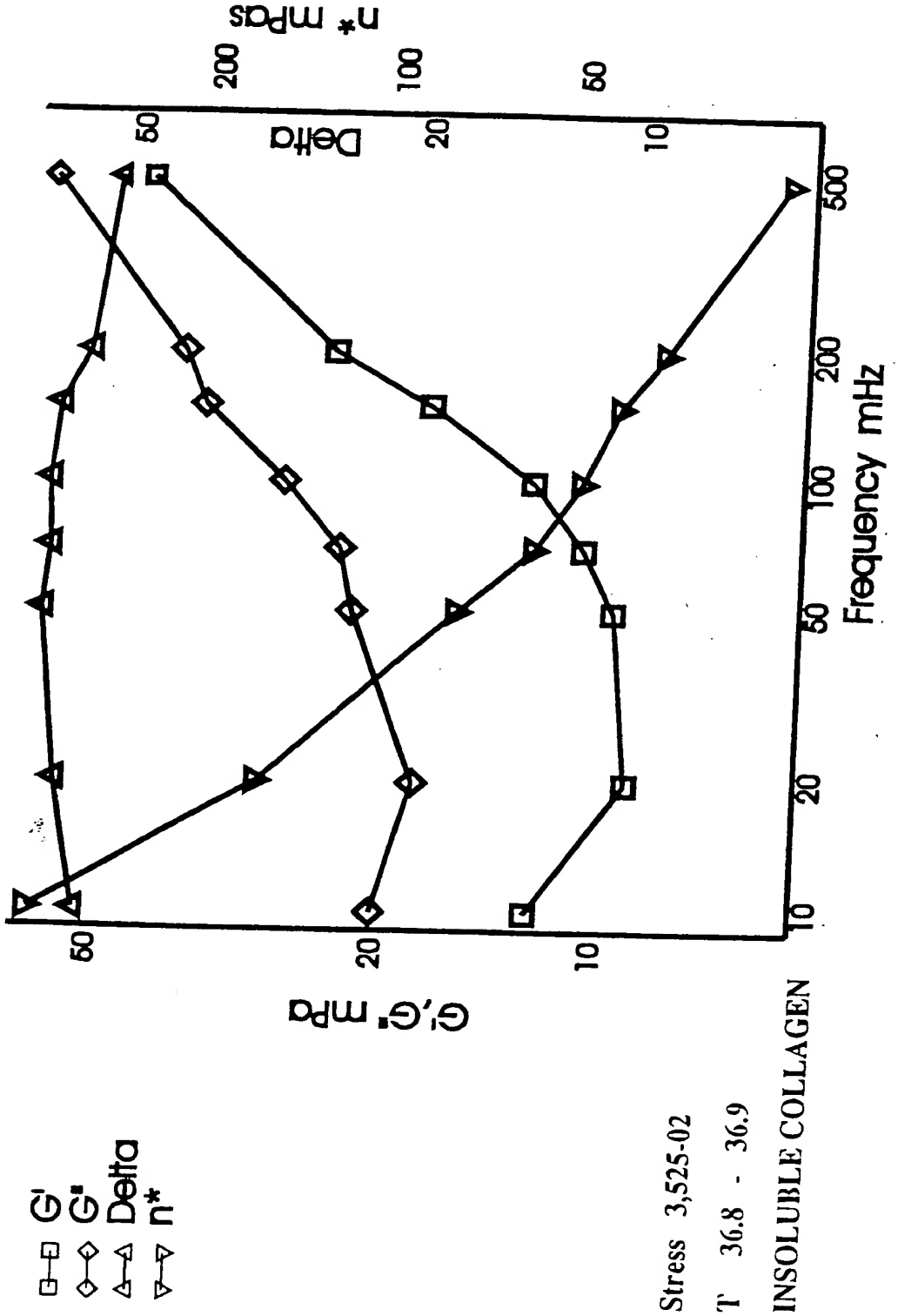

n* mPas
200
100
50
Delta
50
20
10
G',G" mPa
50
20
10
Frequency mHz
10
20
50
100
200
500
G'
G"
Delta
n*
Stress 3,525-02
T 36.8 - 36.9
INSOLUBLE COLLAGEN

FIGURE 2

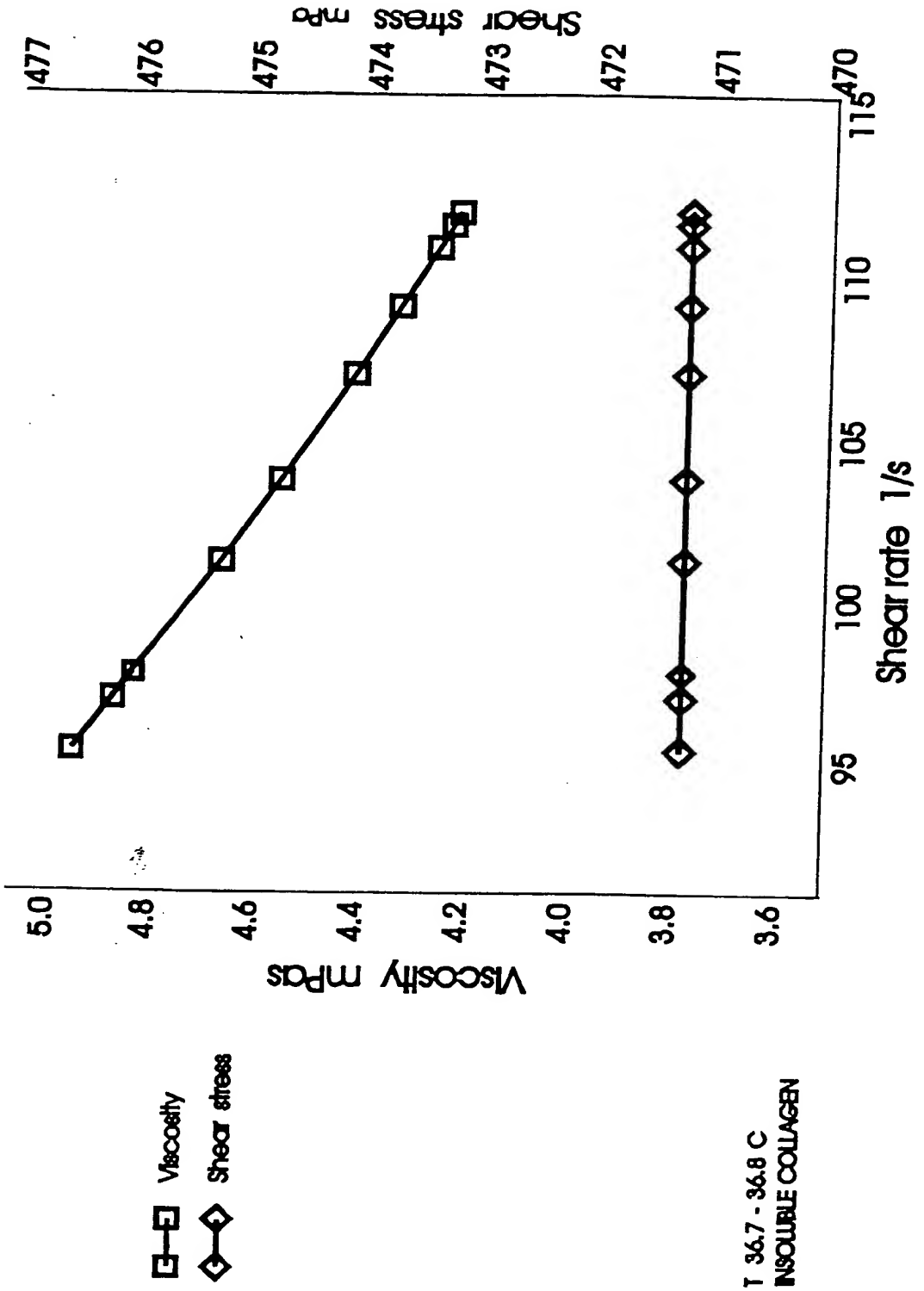
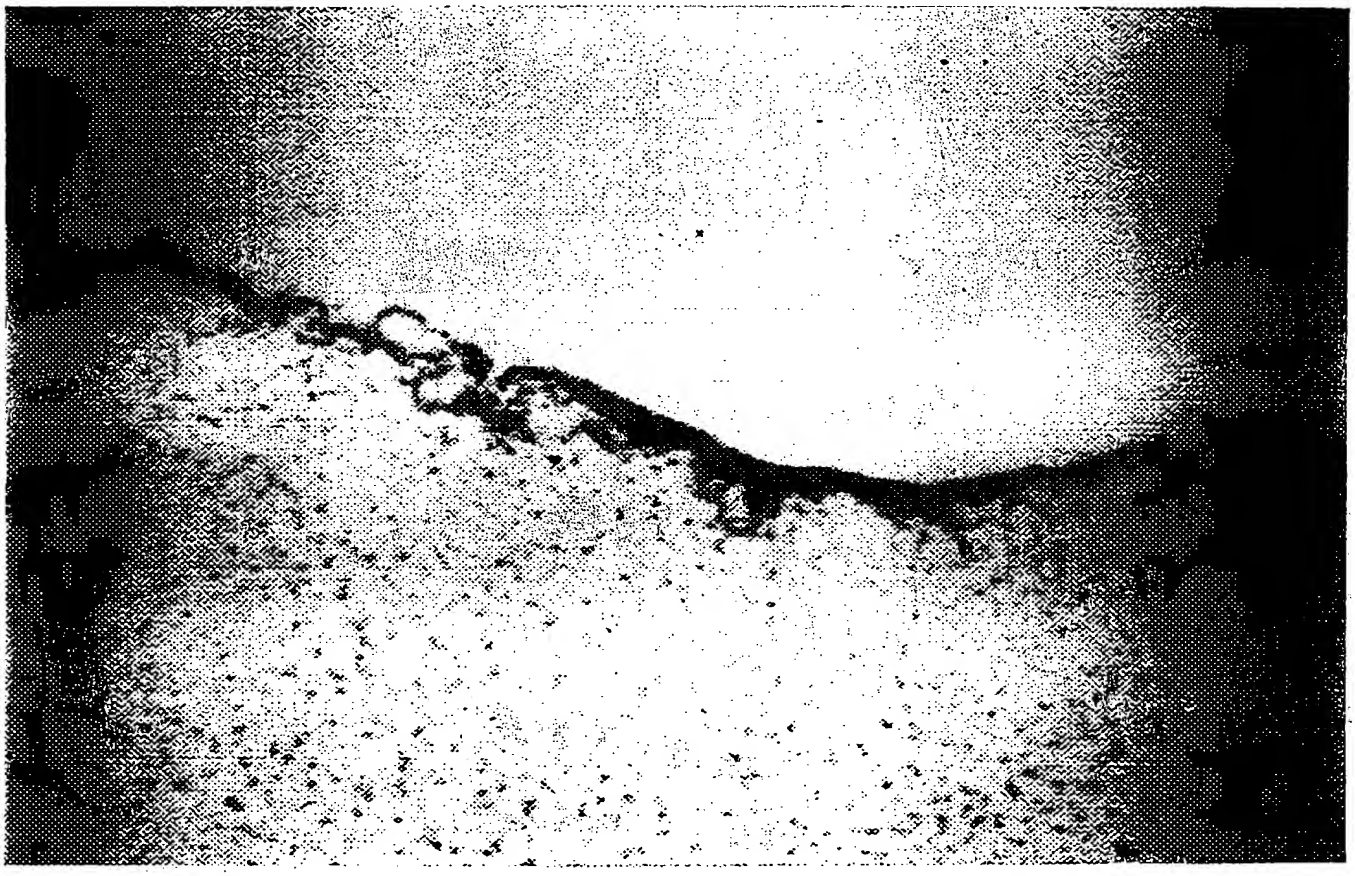

Shear stress mPa
477
476
475
474
473
472
471
470
115
110
105
100
95
Shear rate 1/s
Viscosity mPas
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
Viscosity
Shear stress
T 36.7 - 36.8 C
INSOLUBLE COLLAGEN

FIGURE 3

Fig 4. Collagen sheet on sponge surface.



FIGURRE 4

Fig 5. Arrow indicates collagen layer beneath epithelial cells.

FIGURE 5

Fig 6. Epithelial outgrowth over collagen coated sponge ; surface view. Edge indicated by arrow.

FIGURE 6

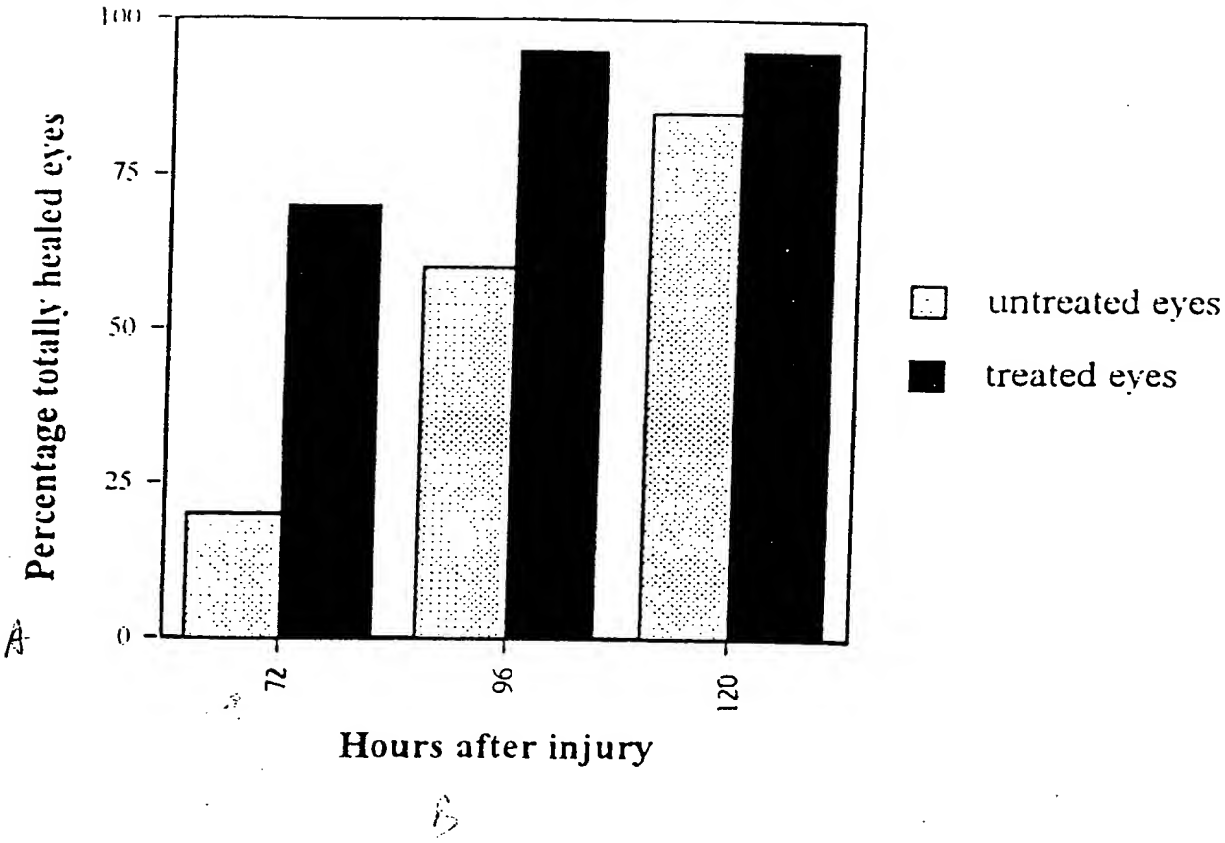

100
75
50
25
0
Percentage totally healed eyes
72
96
120
Hours after injury
untreated eyes
treated eyes

FIGURE 7

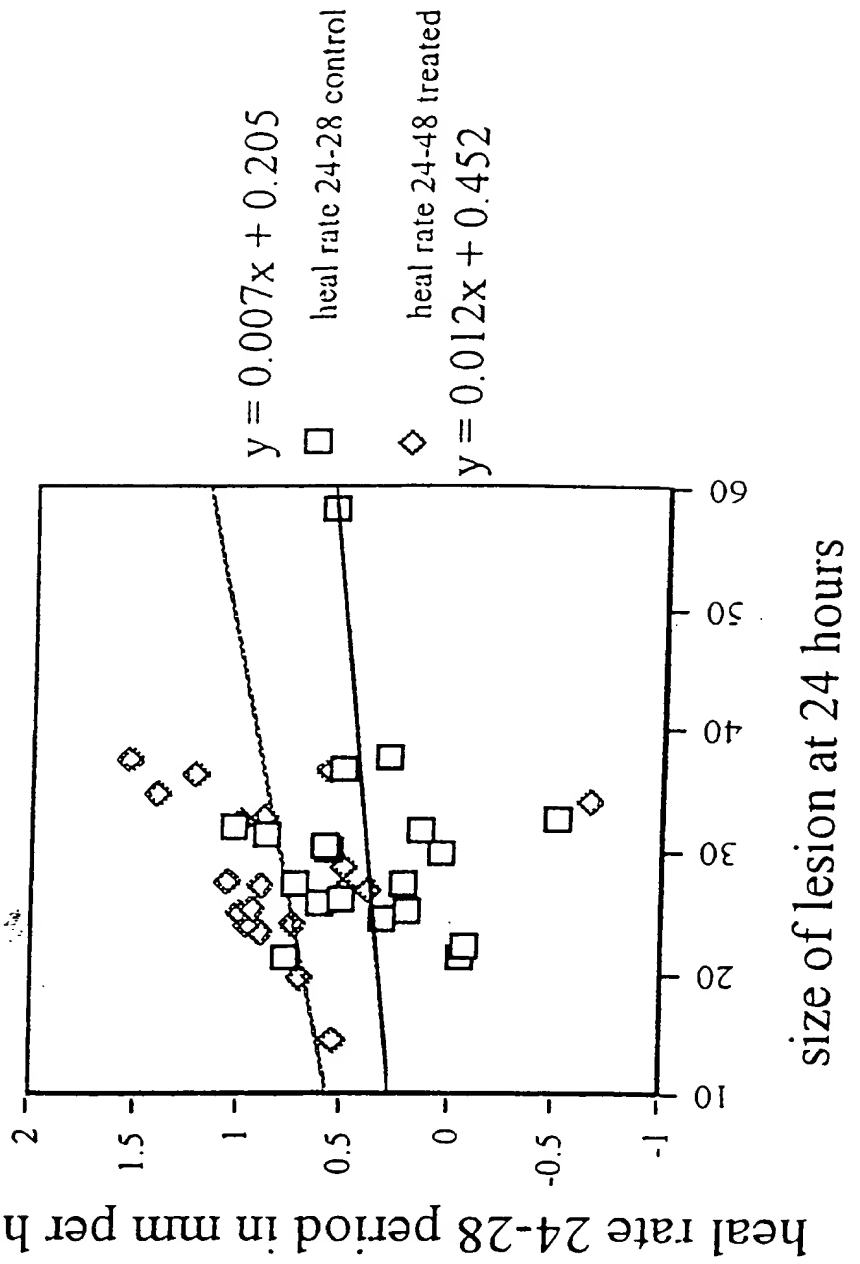

y = 0.007x + 0.205
heal rate 24-28 control
heal rate 24-48 treated
y = 0.012x + 0.452
size of lesion at 24 hours
heal rate 24-28 period in mm per hour
2
1.5
1
0.5
0
-0.5
-1
10
20
30
40
50
60

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00773

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61L 15/32, A61K 9/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61L 15/32, A61K 9/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: (A61L 15/32, A61K 9/70) and "collagen"
DERWENT: (A61L 15/32 A61K 9/70) and "collagen"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4808570 (MICHAELI), 28 February 1989 See whole document	1-30
A	AU 80281/91 (BAUSCH & LOMB INC), 9 January 1990 See whole document	1-30

☐ Further documents are listed in the continuation of Box C

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Date of the actual completion of the international search
8 December 1997

Date of mailing of the international search report
11 DEC 1997

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00773

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		US	4745098	US	4837024		

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